



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: C12N 15/11, A61K 31/70, C07H 21/00 // C12N 9/22	A1	(11) International Publication Number: WO 99/27086 (43) International Publication Date: 3 June 1999 (03.06.99)
(21) International Application Number: PCT/GB98/03500 (22) International Filing Date: 24 November 1998 (24.11.98) (30) Priority Data: 9724916.3 25 November 1997 (25.11.97) GB 9801617.3 26 January 1998 (26.01.98) GB (71) Applicant (for all designated States except US): BRAX GENOMICS LIMITED [GB/GB]; 13 Station Road, Cambridge CB1 2JB (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): SCHMIDT, Günter [DE/GB]; Houghton Manor, Houghton, Cambs PE17 2BQ (GB). THOMPSON, Andrew, Hugin [GB/GB]; 25 Knoll Park, Alloway, Ayr KA7 4EH (GB). (74) Agents: DANIELS, Jeffrey, Nicholas et al.; Page White & Farrer, 54 Doughty Street, London WC1N 2LS (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: CHIMERIC ANTISENSE OLIGONUCLEOTIDES AGAINST TNF-ALPHA AND THEIR USES		
(57) Abstract <p>An agent capable of inhibiting expression of TNF-α in TNF-α expressing cells, which agent comprises an oligonucleotide comprising 8 to 18 bases, at least 8 of which are contiguous bases from one of the following sequences: AGAGTCCCCGGT; GGGAGAGAGGGG; ACCTTTCCTGTG; TACTTACATAAA; TAAACCCTCTGG; CTCCTCCGCGAG; AATTCAACAGAT; CGGATCATGCTT; TGCAAA-CATAAA; ACATAAATAGAG; TCACAAGTGCAA; TGCTTTCAGTGC; and TTCTTTCCTAAG.</p>		

- 1 -

CHIMERIC ANTISENSE OLIGONUCLEOTIDES AGAINST TNF-ALPHA AND THEIR USES

The present invention relates to an agent capable of inhibiting expression of tumour necrosis factor α (TNF- α) in TNF- α expressing cells and the use of such an agent in therapy of disorders associated with TNF- α .

TNF- α plays an important role in many inflammatory rheumatic diseases (24), and it regulates the expression of several proteins, including the Class I antigens of the major histocompatibility complex (MHC) and cytokines such as interleukin-1 and interleukin-6 (25,26). TNF- α also appears to be essential for normal immune responses, however over-expression can produce destructive effects such as rheumatoid arthritis (27). TNF- α is the cytokine responsible for the induction of HIV-1 expression in ACH-2 cells (28). TNF- α also induces the production of transcription factors that bind to the NF-kB enhancer elements within the 5' viral long terminal repeat sequences and thus activating HIV-1 expression.

All these roles of TNF α make it extremely important that effective agents that can target TNF α 's expression directly be found both for use as research tools to understand its activity or as drugs that can inhibit the effects of over-expression of the TNF α gene product in disease.

The inhibitory effect of oligonucleotides (ONs) on gene expression has been known for some time. Stephenson and Zamecnik first proposed that ONs could be used for therapeutic purposes. They demonstrated that a 13mer ON complementary to a region of the Rous Sarcoma virus could inhibit growth of the virus in culture (29,30). Since then the first observations of activatory effects of ONs have also been observed. PCT/GB96/02275 describes a library approach for identifying antisense binding sites using TNF- α as an example.

Mechanisms by which oligonucleotides exert their effects:

- 2 -

Oligonucleotides can interact with cellular nucleic acids at numerous levels, such as with:

- Genomic DNA
- Pre mRNA
- Heteronuclear ribonucleoproteins (hnRNPs)
- Cytoplasmic mRNA
- Cytoplasmic ribonucleoproteins, tRNAs, etc.
- Nucleic Acid binding proteins.

Various mechanisms of action have been proposed but very few have been demonstrated categorically. For the purposes of controlling gene expression specifically, targetting gene mRNAs seems to be most likely to be viable, although various approaches to regulation at the level of genomic DNA are showing some promise. Two approaches to regulating mRNAs are available, using antisense RNAs, preferably expressed in vivo by some vector, which act by blocking access to the mRNA of ribosomes or poly-adenylation enzymes. The alternative approach is using oligodeoxynucleotides (ODNs) which have to be delivered externally. These have been shown to inhibit mRNAs on hybridisation by inducing RNase H mediated degradation of the RNA strand (31,32). The specific degradation of only the RNA in the hybrid duplex means the ODN can act catalytically to hybridise and facilitate degradation of further mRNA molecules.

According to one aspect, the present invention provides an agent capable of inhibiting expression of TNF- α in TNF- α expressing cells, which agent comprises an oligonucleotide comprising 8 to 18 bases, at least 8 of which are contiguous bases from one of the following sequences: AGAGTTCCTGGT; GGGAGAGAGGGG; ACCTTTCCTGTG; TACTTACATAAA; TAAACCCTCTGG; CTCCTCCGCGAG; AATTCAACAGAT; CGGATCATGCTT; TGCAAACATAAA; ACATAAATAGAG; TCACAAGTGCAA; TGCTTTCAGTGC; and TTCTTTCCTAAG.

Preferably, the oligonucleotide comprises an antisense oligonucleotide capable of hybridising to TNF- α mRNA in its native conformation.

- 3 -

Preferably the oligonucleotide is no more than 12 bases long. More preferably the oligonucleotide comprises one of the above sequences. In a particularly preferred embodiment, the oligonucleotide is a chimaeric oligonucleotide which may be made synthetically using any commonly-available oligonucleotide sequence synthesiser.

The oligonucleotide is preferably protected against nuclease attack so as to minimise degradation in the cell and increase its stability. This is particularly important in the design of an antisense compound for therapeutic use. Protection against exonuclease attack may be achieved by protecting one or preferably both ends of the oligonucleotide, for example by reverse T or any other well-known method. Selection of the nucleotides constituting the recognition and flanking regions may also contribute to stability against nuclease because some nucleotides are more nuclease-resistant than others.

Preferably, each chimaeric oligonucleotide comprises two flanking regions, one on either side of the recognition region. In this way, the recognition region may be thought of as a "window" flanked by the two flanking regions so as to form with the mRNA a substrate for the duplex-cutting RNAase. In a preferred embodiment, each of the flanking regions is protected against exonuclease attack, preferably by reverse T. A preferred duplex-cutting RNAase is RNAase H, advantageously endogenous RNAase H (Ref 23).

The nucleotides constituting the recognition region are either modified or unmodified nucleotides and are preferably deoxyribonucleotides or phosphorothioate deoxyribonucleotides (see Figure 4c). These nucleotides are recognisable by RNAase H when hybridized to mRNA. Typically, the recognition region comprises at least four nucleotides, preferably 5 to 10 nucleotides. In a particularly preferred embodiment, the recognition region comprises five or six nucleotides.

- 4 -

The nucleotides constituting the flanking region are chemically modified so as to increase the binding constant of the oligonucleotide for hybridization to the target mRNA and preferably to increase stability of the oligonucleotide *in vivo*. For a particular antisense oligonucleotide, the efficiency of hybridization to mRNA is a function of concentration. Thus, to improve hybridization at a given concentration, the stability of the hybrid duplex must be increased. A number of chemical modifications can be introduced into the oligonucleotide for this purpose and these fall into three broad classes (see also Figure 1, regions 1, 2 and 3):

Sugar Modifications

Various modifications to the 2' position in the sugar moiety may be made. For example, both 2'-O methyl oligoribonucleotides and 2'-O allyl oligoribonucleotides may be useful (see references 1 and 2 and see also Figure 2a and b). These analogues do not form hybrid duplexes with RNA which are substrates for RNAase H. In a particularly preferred embodiment of the present invention, two flanking regions, each having four or five of one of the modified sugar-containing oligoribonucleotides, flank a window region of four or five normal deoxyribonucleotides. The window region will thereby allow cleavage of the mRNA and the sugar-modified flanking regions increase the binding of the chimaeric oligonucleotide to the mRNA. Other 2' sugar modifications which may be used include F-substituted and NH₂-substituted oligoribonucleotides (see Figure 2c and 2d and references 3 and 4).

Various further modifications to the 2' position in the sugar moiety may be made. The sugar may be replaced by a different sugar such as hexose or the entire sugar phosphate backbone can be entirely replaced by a novel structure such as in peptide nucleic acids (PNA). PNA may be the ideal choice for antisense forms used to bring other entities into proximity with the TNF α RNA as it forms duplexes of the highest thermal stability of any analogues so far discovered.

- 5 -

- C.J. Guinosso, G.D. Hoke, S.M. Freier, J.F. Martin, D.J. Ecker, C.K. Mirabelle, S.T. Crooke, P.D. Cook, *Nucleosides Nucleotides* 10, 259 - 262, 1991.
- M. Carmo-Fonseca, R. Pepperkok, B.S. Sproat, W. Ansorge, M.S. Swanson, A.I. Lamond, *EMBO J.* 7, 1863 - 1873, 1991.
- P.E. Nielsen, *Annu. Rev. Biophys. Biomol. Struct.* 24, 167 - 183, 1995.

Base Modifications

The chemically-modified nucleotides constituting the flanking region may be modified in the base moiety. The propyne analogues of dT and dC, 5-propynyl deoxyuridine (see Figure 3a) and 5-propynyl deoxycytidine (see Figure 3b), both increase the duplex hybridization temperature and stabilize the duplex. This stabilization may be due to increased strength of hydrogen bonding to each Watson-Crick partner or increased base stacking (or both). 2-amino adenine is an analogue of dA (see Figure 3c) and also increases the stability of the duplex. This may be due to the formation of a third hydrogen bond with thymine. The 2-amino adenine-thymine base pair is intermediate in stability between a G.C and a A.T base pair.

Numerous modifications can be made to the standard Watson-Crick bases. The following are examples of modifications that should normalise base pairing energies to some extent but they are not limiting:

- The adenine analogue 2,6-diaminopurine forms three hydrogen bonds to thymine rather than two and therefore forms more stable base pairs.
- The thymine analogue 5-propynyl dU forms more stable base pairs with adenine.
- The guanine analogue hypoxanthine forms two hydrogen bonds with cytosine rather than three and therefore forms less stable base pairs.

These and other possible modifications should make it possible to compress the temperature range at which short oligonucleotides can hybridise specifically to their complementary sequences.

Phosphate Modifications

The chemically-modified nucleotides constituting the flanking region may be modified in the phosphate moiety. Under certain conditions such as low salt concentration, analogues such as methylphosphonates (Figure 4a), triesters (Figure 4b) and phosphoramidates (Figure 4e) have been shown to increase duplex stability. The hybrid duplexes are not necessarily substrates for RNAase H. Further phosphate modifications include phosphorodithirates (Figure 4d) and boranophosphates (Figure 4f), each of which increase the stability of oligonucleotides. A phosphorothioate modification is also useful in the flanking region. Although this is a substrate for RNAase H, a phosphorothioate-modified flanking region prevents non-specific hybridisation by preventing the formation of secondary structure in the oligonucleotide.

Isosteric replacement of phosphorus by sulphur gives nuclease resistant oligonucleotides (see reference 14). Replacement by carbon at either phosphorus or linking oxygen is also a further possibility (see Figure 5).

In use, it is believed that the chimaeric oligonucleotide of the invention acts as an antisense compound by specifically binding to target TNF- α mRNA at an antisense binding site so that cleavage or cutting of the mRNA by a duplex-cutting RNAase takes place there. The chimaeric oligonucleotide will bind to the target mRNA to form a duplex. The recognition region is recognised by a duplex-cutting RNAase. The flanking region renders the duplex sufficiently stable to enable the RNAase to cut the mRNA in the duplex efficiently. Once cut, the mRNA and the oligonucleotide detach thereby leaving the oligonucleotide to bind a further mRNA. In this way, the chimaeric oligonucleotide acts catalytically.

Useful forms of the molecules:

The mechanism of action of these molecules is through mediation of cleavage of TNF α by RNase H. This enzyme requires the presence of a normal phosphodiester backbone or a phosphorothioate backbone to allow recognition of the agents in the region of the cut site but in the region flanking the cut site other backbone chemistries might be appropriate such as methylphosphonates, 5' to 5' linkages, or peptide bonds in order to:

- prevent exonuclease degradation of the molecules.
- allow increased affinity of the antisense molecule for its target.

Modifications of bases can also be tolerated and would be beneficial for similar reasons as for backbone modifications.

Conceivably other sequences might be useful to flank the antisense sequences themselves. Since these sequences mediate cleavage of their target RNA molecules it would be important to ensure they were highly specific to their target. The results of S.T.Cload and A.Schepartz, (J. Am. Chem. Soc. 116, 437 - 442, 1994) show that pairs of oligonucleotides can be tethered by a flexible linker molecule. This allows one to use pairs of antisense agents both targeted against a specific gene. Conceivably one might use an oligonucleotide that does not mediate cleavage but that binds with high specificity to the target or one could tether the agent to a TNF α ribozyme such as those described in WO 96/39499.

RNA forms of the molecules or forms of the molecules with backbones not recognised by RNase H will bind the TNF α RNA but will not mediate degradation hence these can be used to bring other agents into proximity with the TNF α RNA or to act as gene specific recognition effectors for a ribozyme.

A method by which the TNF- α mRNA antisense binding site may be identified is disclosed in International Patent Application WO97/10332. That application provides use of an oligonucleotide

- 8 -

library in a method of identifying an antisense binding site in a target mRNA, such as TNF- α or a portion of the sequence thereof. The oligonucleotide library comprises a plurality of distinct nucleotide sequences, each having a common length in the range 7 to 20 bases, preferably 10 to 20 bases, and each of which comprises a substrate for a duplex-cutting RNAase if hybridised to the mRNA, which library is generated randomly, or generated from information characterising the sequence of the target mRNA, so that substantially all nucleotide sequences of said common length which are present as sub-sequences in the target mRNA are present in the library. The nucleotide sequences may comprise modified nucleotides, such as phosphorothioates, as described herein. The nucleotide sequences may be chimaeric or non-chimaeric.

In one embodiment, the library is generated randomly by means of an oligonucleotide sequence synthesizer. An aim of generating the sequences randomly is that substantially all possible nucleotide sequences of the specified length are generated. For a sequence of 10 bases in length (a 10-mer), 4^{10} distinct nucleotide sequences would need to be generated to cover all possibilities. This works out as approximately 10^6 distinct nucleotide sequences. For a 15-mer, the library would need approximately 10^9 to 10^{10} nucleotide sequences. Each nucleotide sequence will have a common length (i.e. they will all be 10-mers or will all be 11-mers, etc.). Any commonly-available oligonucleotide sequence synthesizer may be used for this purpose such as supplied by Applied Biosystems. All four possible bases are fed into the machine with an appropriate program using suitable nucleotides or modified nucleotides.

In an alternative embodiment, instead of generating the nucleotide sequences randomly they are generated from information characterising the sequence of the target mRNA. The sequence of the target mRNA needs to be known and can then be programmed into the oligonucleotide sequence synthesizer. For example, in the case of a gene which produces an mRNA of 450 nucleotides, a

- 9 -

library of 15-mers would be produced with a total of 436 distinct nucleotide sequences (i.e. length of mRNA minus length of nucleotide sequence plus 1). In this way, all potential sub-sequences of the mRNA would be represented in the library. This is advantageous over the random generation of the library because there is no dilution of potentially useful nucleotide sequences by randomly generated sequences not present in the target mRNA. A further way of ensuring that all sub-sequences of the mRNA are present in the library is to produce, in the case of an mRNA of 450 nucleotides, a library of 30 15-mers (i.e. length of mRNA divided by the length of the nucleotide sequence).

In a further aspect, WO97/10332 provides a method of identifying an antisense binding site in a target mRNA, such a TNF- α or a portion thereof which comprises:

- 1) incubating with the target mRNA an oligonucleotide library and a duplex-cutting RNAase under conditions to produce target mRNA cut at the antisense binding site; and

- 2) identifying the antisense binding site from the position of the cut in the mRNA; wherein the oligonucleotide library comprises a plurality of distinct nucleotide sequences, each having a common length in the range 7 to 20 bases, preferably 10 to 20 bases, and each of which comprises a substrate for the duplex cutting RNAase if hybridized to the mRNA; and wherein the oligonucleotide library is generated randomly, or generated from information characterising the sequence of the target mRNA, so that substantially all nucleotide sequences of such common length which are present as sub-sequences in the target mRNA are present in the library.

Use of an oligonucleotide library in this manner enables identification of one or more antisense binding sites in a target mRNA and such identification can be achieved very rapidly in comparison with known methods. No information about the three-dimension structure of the mRNA is required because the identification of the antisense binding sites is empirical. Incubation of the target mRNA with the oligonucleotide library

- 10 -

and the duplex-cutting RNAase can, by suitable variation of the reaction conditions, produce target mRNA cut at one or more antisense binding sites. This is because the library will contain one or more oligonucleotides which are complementary to such binding sites and will bind thereto under appropriate conditions to form a duplex. The duplex acts as a substrate for the duplex cutting RNAase. When the mRNA is cut at the binding site the oligonucleotide is released and is thereby made available for further binding. The oligonucleotide therefore acts catalytically and the duplex-cutting RNAase acts enzymatically. The duplex-cutting RNAase is separate from the oligonucleotide library and is preferably from a cell extract. Preferably, the duplex-cutting RNAase is RNAase H. The target mRNA is also preferably from a cell extract. Advantageously, therefore, both RNAase H and mRNA are present in the same cell extract with which the oligonucleotide library is incubated.

The position of the cut in the mRNA may be determined by sequencing isolated cut target mRNA. Preferably, the cut target mRNA is amplified prior to isolation, for example by reverse transcription and polymerase chain reaction.

Once the antisense binding site is identified from the position of the cut in the mRNA and sequenced, an antisense oligonucleotide may be synthesized which is capable of binding to the site. In this way, a method is provided for the production of an antisense oligonucleotide. Thus, a chimaeric oligonucleotide of the type discussed above can be obtained. An important use of such a chimaeric oligonucleotide is as a therapeutic agent capable of hybridising to a specific antisense binding site in a target mRNA. The nucleotide sequence of the chimaeric oligonucleotide needs to be specific to the antisense binding site for this purpose.

Therapeutical Applications

The present invention further provides a composition comprising an antisense oligonucleotide as described above, for use in the

- 11 -

treatment of a disorder associated with expression of TNF- α . The composition may further comprise a duplex cutting enzyme such as RNAase H. However, for many applications, no exogenously administered duplex cutting enzyme is required because the antisense oligonucleotide can make use of endogenous RNAase H.

The composition may further comprise one or more further antisense oligonucleotides as described above, wherein each antisense oligonucleotide is different from one another. In this way, by using two or more such antisense oligonucleotides the target TNF- α mRNA may be blocked or destroyed more effectively.

The composition according to the present invention may be used to treat a variety of disorders associated with expression of TNF- α , particularly those associated with overexpression or uncontrolled expression of TNF- α . For example, septic shock such as following infection with gram negative bacteria is a most dramatic example of uncontrolled TNF- α response leading to host cell damage. Over production of TNF- α may play a roll in various pathological conditions including cachexia, autoimmune disorders and meningococcal septicemia, and may have a role in initiation/progression of inflammatory processes including a variety of pulmonary inflammatory disorders, such as acute and interstitial inflammation such as ARDS, extrinsic allergic alviolititis and infectious pneumonias and rheumatoid arthritis. TNF- α possesses the ability to induce or suppress the expression of a vast number of genes, including those for growth factors, other cytokine mediators, transcription factors such as NF K Band acute phase proteins and as such will have a multitude of biological actions.

Compositions according to the present invention may also be used in the treatment of inflammatory skin disorders such as psoriasis, eczema and ultraviolet erythema. The use of these compositions, possibly together with other antisense oligonucleotides may have value in the treatment of atopic

eczema, psoriasis vulgaris and ultraviolet-B erythema. A further disorder capable of treatment is graft versus host disease.

Compositions of the type described above may incorporate an effective amount of the antisense oligonucleotide, optionally together with suitable diluents, preservatives, solubilisers, emulsifiers, adjuvants and/or carriers useful for therapy. Such compositions are liquids or lyophilised or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCL, acetate phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilising agents (e.g. Thimerosal, benzyl alcohol), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the oligonucleotide, complexation with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, polyvinyl pyrrolidone, etc. or into liposomes, microemulsions, micelles, unilamellar or multimellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance of the oligonucleotide. Other ingredients optionally may be added such as antioxidants, e.g., ascorbic acid; low molecular weight (less than about ten residues) polypeptides, i.e., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; amino acid; such as glycine, glutamine acid, aspartic acid, or arginine; chelating agents such as EDTA; and sugar alcohols such as mannitol or sorbitol. Possible sustained release compositions include formulation of lipophilic depots (e.g. fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., polyoxamers or polyoxamines) and oligonucleotides coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Further, specific nucleotide sequences may be added to target the

oligonucleotides of this invention to the nucleus, cytoplasm or to specific types of cells. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

Suitable topical formulations include gels, creams, solutions, emulsions, carbohydrate polymers, biodegradable matrices thereof; vapours, mists, a aerosols, or other inhalants. The oligonucleotides may be encapsulated in a wafer, wax, film or solid carrier, including chewing gums. Permeation enhancers to aid in transport to movement across the epithelial layer are also known in the art and include, but are not limited to, dimethyl sulfoxide and glycols.

Synthesis of Oligonucleotides:

The invention also embodies methods of production of the compounds and RNA molecules described above comprising the steps of: (a) ligating into a transfer vector comprised of DNA, RNA or a combination thereof a nucleotide sequence corresponding to said compound; (b) transcribing the nucleotide sequence of step (a) with RNA polymerase; and (c) recovering the compound. The invention also includes transfer vectors, bacterial or phage, comprised of RNA or DNA or a combination thereof containing a nucleotide sequence which on transcription gives rise to the compounds or RNA molecules described above.

Further, many methods have been developed for introducing cloned eukaryotic DNAs into cultured mammalian cells (Sambrook et al. Molecular Cloning: A Laboratory Manual 2ed. Cold Spring Harbor Laboratory Press 1989): Calcium phosphate or DEAE-dextran mediated transfection; Polybrene; Protoplast fusion; Electroporation; and direct microinjection into nuclei.

Synthetic preparations of mRNA are well known (see Sambrook et al. Molecular Cloning: A Laboratory Manual 2ed. Cold Spring

- 14 -

Harbor Laboratory Press 1989). Mixed DNA/RNA oligomers with modified base pairs for the TNF- α ribozyme can be prepared by commercially available DNA synthesisers such as those produced by Applied Biosystems, Biosearch, or Milligen (see, e.g., Perrault et al., Nature, 344:565 - 567 (1990, for derivatives Uhlmann, E. and Peyman, A. Chemical Reviews (1990) 90:543 - 584, H-phosphonate monomers see Agrawal et al. U.S. Patent No. 5, 149,798).

Details and reviews on the construction of oligonucleotides are available in numerous up to date texts:

- Gait, M.J. editor, 'Oligonucleotide Synthesis: A Practical Approach', IRL Press, Oxford, 1990
- Eckstein, editor, 'Oligonucleotides and Analogues: A Practical Approach', IRL Press, Oxford, 1991
- Kricka, editor, 'Nonisotropic DNA Probe Techniques', Academic Press, San Diego, 1992
- Haugland, 'Handbook of Fluorescent Probes and Research Chemicals', Molecular Probes, Inc., Eugene, 1992
- Keller and Manack, 'DNA Probes, 2nd Edition', Stockton Press, New York, 1993
- Kessler, editor, 'Nonradioactive Labeling and Detection of Biomolecules', Springer-Verlag, Berlin, 1992.

The present invention will now be described in further detail, by way of example only, with reference to the accompanying drawings in which:

Figure 1 shows a generalised structure for nucleotides;

Figure 2 shows a generalised structure for sugar moieties present in nucleotides;

Figure 3 shows generalised structures of modified base moieties in nucleotides;

Figure 4 shows generalised structures of modified phosphate moieties in oligonucleotides;

Figure 5 shows isosteric modifications of the phosphate moieties in oligonucleotides;

- 15 -

FIGURE 6 shows histogram (mean \pm s,d) of production (pg/ml) of TNF- α by MM6 cells in culture.

FIGURE 7 shows a histogram (mean \pm s,d) of production (pg/ml) of TNF- α by MM6 cells in culture.

Nucleotide modifications

Figure 1 shows a generalised structure for nucleotides in which base 2 is connected to sugar 1 and phosphate 3 links the sugar to the next sugar in the sugar phosphate backbone. According to Figure 2, substituent group A may be O-alkyl, aryl or alkaryl (in particular O-Me) (see Ref 1). Alternatively, substituent group A may be O-allyl (Ref 2), F (Ref 3), or NH₂ (Ref 4).

The bases shown in Figures 3a, 3b and 3c are discussed respectively in References 5, 6 and 7. Referring to Figure 4, various phosphate modifications may be made, as summarised in the Table I below.

x	y	Ref
CH ₃	O	8
OR	O	9
S	O	10
S	S	11
NHR	O	12
BH ₃	O	13

R = any alkyl

Referring to Figure 5, the phosphate moiety may be replaced in accordance with the following Table II

X	Y	Z	Ref
O	CH ₂	O	15
O	CH ₂	S	16
S	CH ₂	O	17
O	C=O	O	18
O	C=O	NH	19

- 16 -

CH ₂	NH	O	20
CH ₂	NCH ₃	O }	
CH ₂	O	NCH ₃ }	21

Example 1 IN VIVO (Cell based) experimentsExperiment 1 - Cell uptake

Experiments were performed to ensure that cellular uptake of oligodeoxyribonucleotides occurred before initiating experiments to establish biological efficacy.

i) HACAT cells, a keratinocyte cell line (Petra Boukamp et al), were grown in culture to approximately 80% confluence using a standard methodology (Groves 1993), then transferred to a 48 well tissue culture plate and allowed to grow for 24 hours.

ii) The cultured HACAT cells were transferred at a concentration of 2×10^5 cells per ml to eight well tissue culture slides and allowed to grow for 24 hours. Two fluorescently labelled antisense DNA compounds were added to the culture media at concentrations of 0.5, 5, 50, and 100 μ M. Cells were observed at 3, 4 and 5 hours by fluorescence microscopy.

V7406 is a chimaeric 12mer oligonucleotide with 3 phosphorothioate backbone linkages at both the 3' and 5' termini. The central linkages are normal phosphate linkages.

V7407 is a chimaeric 12mer oligonucleotide with 3 methyl-phosphonate backbone linkages at both the 3' and 5' termini. The central linkages are again normal phosphate linkages.

In both cases an arbitrary sequence was used (5'-TCTCAAGGGCCA-3').

iii) The above experiment was repeated stimulating the cells with V7406 and V7407 at 10 and 50 μ M for 2, 4, 8 and 24 hours and

- 17 -

for 24 hours at 4°C. Control cells were incubated with fluorescent label (FAMDYE) for the same time points.

Results

i) Cells incubated with V7406 demonstrated positive labelling with an increase in uptake correlating with increasing concentration and time. Positive labelling was confined to the cell nucleus however, for all treatments, some cells showed no labelling. None of the cells incubated with V7407 were fluorescently labelled.

ii) V7406 produced patches of localised labelling within keratinocyte nuclei. Not all cells were equally stained but could be grouped roughly into three populations, strongly labelled, weakly labelled and negative. Optimal labelling was achieved using a concentration of 50µm V7406 for 8 hours. V7407 did not produce positive labelling. FAMDYE produced either none or only very weak staining of keratinocytes.

REFERENCE

Expression of Selection ligands by cutaneous squamous cell carcinoma. Groves RW, Allen MH, Ross EL et al. Am. J. Pathol., 143(4), 1220-1225, 1993.

Petra Boukamp et al. J. Cell Biol., 106, 761-771, March, 1988.

Experiment 2 - Biological Efficacy I

Procedure for assay of antisense inhibition of TNFα

1. At study time 0:00 hrs human monocyte cells (Mono-Mac 6 cell line 'MM6') passage number 21 were placed in suspension in 24 well cell culture plates at a density of 0.4 million/ml (Total volume in each well of 0.4 ml). The cell culture media comprised freshly prepared RPMI media (Gibco Life Technologies) supplemented with 10% serum. The cells were placed in 5% CO₂ atmosphere/air atmosphere in a humidified 37°C incubator.

- 18 -

2. At study time 22:00 hrs aliquotes of oligos were spiked into the culture wells of the 24-well plate to achieve a final concentration of 40 uM. For each oligo stock solution supplied by Oswell DNA Service, Southampton, UK (oligos supplied in sterile water) the volume of stock required to be spiked into each culture well to achieve a final oligo concentration of 40 uM will vary as a function of their synthesis. However, the final amount of sterile water added to the culture wells is kept constant from one oligo to another by the further addition of an aliquote of sterile water to result in a final volume of sterile water of 0.17 ml in a total final volume in each culture well of 0.588 ml.

3. At 25:00 hrs the cells were removed from the incubator and endotoxin (LPS - Sigma catalogue L3024) and phorbol myristate acetate (PMA - Sigma catalogue P8139) added to each well to stimulate production of $\text{TNF}\alpha$.

Specifically, LPS (stock at 20 ug/ml in saline) is added to achieve a final conc of 50 ng/ml. The stock is diluted in RPMI supplemented with 10% serum with the volume added to each well equating to 12 uL.

Specifically, PMA (stock at 100 ug/ml in DMSO) is added to achieve a final conc of 5 ng/ml. The stock is diluted in RPMI supplemented with 10% serum with the volume added to each well equating to 5uL.

The total volume in each well at study time 25 hrs is therefore 0.588 mL comprising 400 uL original media with cells + 170 uL (oligo/sterile water adjustment) + 17 uL LPS and PMA.

4. The cell culture plates are then transferred back to the incubator and left for 3 hrs. At study time 28 hrs the media from each well (inc. cells) were transferred to eppendorf microcentrifuge and spun at 2000 g for 4 min to pellet intact

- 19 -

cells. The supernatant was removed and frozen at -80°C to await analysis.

5. Samples were thawed at 4°C and analysis undertaken using BIOTRAK ELISA kit for human TNF α from Amersham (product RPN 2758) (Batch 99809 expiry July 4th 1997). Time of harvesting to analysis did not exceed 21 days. All samples for given MM6 study were analysed at the same time by ELISA. The measurement filter for ELISA was set at 450 nm and the reference at 620 nm.

6. RESULTS of STUDY

Study Treatments (see Table inc. volumes of sterile water/oligo and LPS and PMA added to cells).

All oligos tested were 12-mer phosphorothioate chimeric oligos with x3 phosphorothioate links at 5' and 3' ends. N=5 for each treatment.

Oligo codes refer to OSWELL sequence name. LPS in Figure 6 refers to LPS/PMA stimulated cells.

Codes refer to OSWELL sequence codes for oligo.

TABLE

	Sterile Water	Media	LPS in media	PMA in media	oligo
Untreated (n=5)		17.5 uL	-	-	
LPS/PMA (n=5)	171 uL		12.5 uL	5 uL	-
Sequence 40 uM					
(TB0279) (n=5)	80 uL		12.5 uL	5 uL	91 uL
(TB0280) (n=5)	19 uL		12.5 uL	5 uL	152 uL
(TB0281) (n=5)	73 uL		12.5 uL	5 uL	98 uL
(TB0282) (n=5)	57 uL		12.5 uL	5 uL	114 uL
(TB0283) (n=5)	55 uL		12.5 uL	5 uL	116 uL
(TB0284) (n=5)	74 uL		12.5 uL	5 uL	97 uL
(TB0285) (n=5)	34 uL		12.5 uL	5 uL	137 uL

Sequence Tested (Shown 5' to 3')

TB0279 AGAGTCCCGGT
 TB0280 GGGAGAGAGGGG
 TB0281 ACCTTTCCTGTG
 TB0282 TACTTACATAAA
 TB0283 TAAACCCTCTGG
 TB0284 CTCCTCCGCGAG
 TB0285 AATTCAACAGAT

*SEQUENCE TB0282 produces a significant ($P < 0.05$) 35% decrease in $\text{TNF}\alpha$ production at 40uM compared to LPS control (Figure 6).

Experiment 3 - Biological Efficacy IIProcedure for assay of antisense inhibition of $\text{TNF}\alpha$

1. At study time 0:00 hrs human monocyte cells (Mono-Mac 6 cell line 'MM6') passage number 13 were placed in suspension in 24 well cell culture plates at a density of 0.4 million/ml (Total volume in each well of 0.4 ml). The cell culture media comprised freshly prepared RPMI media (Gibco Life Technologies)

- 21 -

supplemented with 10% serum. The cells were placed in 5% CO₂ atmosphere/air atmosphere in a humidified 37°C incubator.

2. At study time 19:00 hrs aliquotes of oligos were spiked into the culture wells of the 24-well plate to achieve a final concentration of 30 or 10 uM. For each oligo stock solution supplied by Oswell DNA Service, Southampton, UK (oligos supplied in sterile water) the volume of stock required to be spiked into each culture well to achieve a final oligo concentration will vary as a function of their synthesis. However, the final amount of sterile water added to the culture wells is kept constant from one oligo to another by the further addition of an aliquote of sterile water to result in a final volume of sterile water of 0.17 ml in a total final volume in each culture well of 0.588 ml.

3. At 22:00 hrs the cells were removed from the incubator and endotoxin (LPS - Sigma catalogue L3024) and phorbol myristate acetate (PMA - Sigma catalogue P8139) added to each well to stimulate production of TNF α .

Specifically, LPS (stock at 20 ug/ml in saline) is added to achieve a final conc of 50 ng/ml. The stock is diluted in RPMI supplemented with 10% serum with the volume added to each well equating to 12 uL.

Specifically, PMA (stock at 100 ug/ml in DMSO) is added to achieve a final conc of 5 ng/ml. The stock is diluted in RPMI supplemented with 10% serum with the volume added to each well equating to 5uL.

The total volume in each well at study time 25 hrs is therefore 0.588 mL comprising 400 uL original media with cells + 170 uL (oligo/sterile water adjustment) + 17 uL LPS and PMA.

4. The cell culture plates are then transferred back to the incubator and left for 3 hrs. At study time 25 hrs the media from each well (inc. cells) were transferred to eppendorf

- 22 -

microcentrifuge and spun at 2000 g for 4 min to pellet intact cells. The supernatant was removed and frozen at -80°C to await analysis.

5. Samples were thawed at 4°C and analysis undertaken using BIOTRAK ELISA kit for human TNF α from Amersham (product RPN 2758). Time of harvesting to analysis did not exceed 21 days. All samples for given MM6 study were analysed at the same time by ELISA. The measurement filter for ELISA was set at 450 nm and the reference at 620 nm.

6. RESULTS of STUDY

The results are shown in Figure 7.

All oligos tested were 12-mer phosphorthioate chimeric oligos with x3 phosphorothioate links at 5' and 3' ends. N=5 for each treatment.

LPS in Figure 7 refers to LPS/PMA stimulated cells. UNTREAT in the Figure refers to unstimulated cells. In the Figure, where the concentration of a sequence is not given in brackets, the concentration was 30 pg/ml.

Sequence Tested (Shown 5' to 3')

Z8902	TGCTTTCAGTGC
Z8900	TCACAAGTGCAA
Z8899	ACATAAATAGAG
Z8898	TGCAAACATAAA
Z8896	CGGATCATGCTT
Z8903	TTCTTTCCTAAG
Z8897	TACTTACATAAA
Z8895	TCAGTGCTCATG
Z8901	ACCTTTCCTGTG
TB0279	AGAGTTCCCGGT
Y0080	AAATACATTCAT

- 23 -

The TNF production in Experiment 3 was lower than that in Experiment 2. As TNF production increases then the ability to detect the effectiveness of active oligomers is progressively lost. Thus the results of Experiment 3 more clearly demonstrate the effectiveness of the oligomers used in the present invention.

The sequences Z8902, Z8900, Z8899, Z8898, Z8896, and Z8903 at a concentration of 30 pg/ml as well as Z8895 at 30 pg/ml and Z8901 at both 30 and 10 pg/ml produce a marked decrease in TNF α production compared with the LPS control (Figure 7).

References

1. Guinasso, C.J., Hoke G.D., Freier, S.M., Martin, J.F., Ecker, D.J., Mirabelle, C.K., Crooke, S.T., Cook, P.D. Nucleosides Nucleotides (1991) 10 259-262
2. Carmo-Fonseca, M., Pepperkok, R., Sproat, B.S., Ansorge, W., Swanson, M.S., Lamond, A.I. Embo J. (1991), 7, 1863-1873
- 3a Cook, P.D. Anti-Cancer Drug Des. (1991) 6 585-607
- b Wagner, R.W. Nature (1994) 372 333-335
- 4 Uhlmann E. & Payman A, Chemical Reviews 90 (1990) 544
- 5 Wagner, R.W. et al Science (1993) 260 1510-1513
- 6 Fenster, S.D., Wagner, R.W., Froehler, B.C. & CHIN, D.J. Biochemistry (1994) 33 8391-8398
- 7 Chollet, A., & Kawashima, E Nucleic Acids Res (1988) 16 305 -317
- 8 Miller, P.S., Reddy, M.P., Murakami, A., Blake, K.R., Lin, S-B., & Agnis, C.H., Biochemistry (1986) 25 5092-5097
- 9 Letsinger, R.L., Groody, E.P., Lander, N., & Tanaka, T. Tetrahedron (1984) 40 137-143
- 10a Stein, C.A., Subasinghe, C., Shinozuka, K., & Cohen J.S. (1988) Nucleic Acids Res., 16 3209-3221
- b Stec, W.J.; Gratkowski, A.; Koziolkiewicz, M.; Uznanski, B. Nucliec Acids Res., (1991) 19 5883-5888
- 11 Marshall W.S., Caruthers.M. Science (1993) 259 1564-1570

- 25 -

- 12 Froehler, B., NG, P., Matteucci, M.
Nucleic Acids Res. (1988) 16 4831 - 4839
- 13 Sood, A., Shaw, R.B., Spieluogel, B.F.
J. AM. Chem. Soc. (1991) 112 9000-9007
- 14 Milligan, J.F., Matteucci, M.D., Martin, J.C.
J. Med Chem (1993) 36(14) 1923-1937
- 15} Matteucci, M., Lin, K.Y., Butcher, S., Moulds, C.
16} J. Am. Chem. Soc. (1991), 113 7767 7768
- 17 Jones, B., Ling, K.Y., Milligan, J.F., Wadwani, S., Matteucci, M.
J. Org. Chem. (1993) 58 2983-2991
- 18 Gait, M.J., Jones, A.S., Jones, M.D., Shepherd, M.J., Walker, R.T.
J. Chem. Soc. Perkin Trans I (1979) 1389 - 1394
- 19 Stirchak, E.P., Summerton, J.E., Weller, D.D.
J Org Chem (1987) 52 4204-4206
- 20 Vasseur, J.J., Debart, F., Sanghui, Y.S., Cook, P.D.
J Am. Chem. Soc (1992), 114, 4006-4007
- 21 Debart. F., Vasseur, J.J., Sanghui, Y.S., Cook, P.D.
Bioogr. Med. Chem. Lett. (1992) 2 1479-1482
- 22 Lieber A, Strausse M, Molecular & Cellular Biology (1995), 15
23. Field, A.K. and Goodchild, J., Exp. Opin. Invest. Drugs (1995),
4(9), 799-821

- 26 -

24. Shinmei et al., Sem. Arth. Rheum. 18 (suppl. 1): 27 - 32, 1989
25. Beutler & Cerami, Annu. Rev. Biochem. 57:505 - 518 1989
26. Beutler & Cerami, Annu. Rev. Immunol. 7:625 - 655, 1989
27. Brennan et al., Lancet ii 244 - 247, 1989
28. Rosenberg & Fauci, Immunol. Today 11:176-180, 1990.
29. Stephenson and Zamecnik, Proc. Natl. Acad. Sci. USA 75, 285, 1978
30. Stephenson and Zamecnik, Proc. Natl. Acad. Sci. USA 75, 280, 1978
31. R.V.Giles et al, Antisense Research and Development 5, 23 - 31, 1995.
32. J. Shuttleworth and A. Colman, EMBO J. 7, 427 - 434, 1988.

- 27 -

CLAIMS:

1. An agent capable of inhibiting expression of TNF- α in TNF- α expressing cells, which agent comprises an oligonucleotide comprising 8 to 18 bases, at least 8 of which are contiguous bases from one of the following sequences: AGAGTTCCCGGT; GGGAGAGAGGGG; ACCTTTCCTGTG; TACTTACATAAA; TAAACCCTCTGG; CTCCTCCGCGAG; AATTCAACAGAT; CGGATCATGCTT; TGCAAACATAAA; ACATAAATAGAG; TCACAAGTGCAA; TGCTTTCAGTGC; and TTCTTTCCTAAG.
2. An agent according to claim 1, wherein the oligonucleotide is no more than 12 bases long.
3. An agent according to claim 1 or claim 2, wherein the oligonucleotide has one of the sequences set out in claim 1.
4. An agent according to any one of claims 1 to 3, wherein the oligonucleotide comprises at least one modified base.
5. An agent according to claim 4, wherein the oligonucleotide is a chimaeric antisense oligonucleotide.
6. An agent according to claim 5, wherein the chimaeric oligonucleotide is protected against exonuclease attack and comprises:
 - a) a recognition region comprising a sequence of nucleotides which is recognisable by a duplex-cutting RNAase when hybridized to the mRNA, and
 - b) a flanking region comprising a sequence of chemically-modified nucleotides which binds to the mRNA sufficiently tightly to stabilise the duplex for cutting of the mRNA in the duplex by the duplex-cutting RNAase, wherein the nucleotides constituting the flanking region are different from those constituting the recognition region.

- 28 -

7. An agent according to claim 6, wherein the recognition region comprises a sequence of nucleotides which is recognisable by RNAase H when hybridized to the mRNA.
8. An agent according to claim 6 or claim 7, wherein the nucleotides constituting the recognition region are deoxyribonucleotides or phosphorothioate deoxyribonucleotides.
9. An agent according to any one of claims 6 to 8, wherein the recognition region comprises at least four nucleotides.
10. An agent according to claim 9, wherein the recognition region comprises 5 to 10 nucleotides.
11. An agent according to any one of claims 6 to 10, wherein the chemically modified nucleotides constituting the flanking region are 2' modified in the sugar moiety.
12. An agent according to claim 11, wherein the chemically-modified nucleotides are 2'-O methyl ribonucleotides or 2'-O allyl ribonucleotides.
13. An agent according to any one of claims 6 to 10, wherein the chemically-modified nucleotides constituting the flanking region are modified in the base moiety and are selected from 5-propynyl deoxyuridine, 5-propynyl deoxycytidine and 2-amino adenine analogues.
14. An agent according to any one of claims 6 to 10, wherein the chemically-modified nucleotides constituting the flanking region are modified in the phosphate moiety and are selected from methyl phosphonate, triester, phosphoramidate, phosphorothioate, phosphorodithirite and boranophosphate analogues.
15. An agent according to any one of claims 6 to 14, wherein the nucleotide sequences each comprise two flanking regions, one on either side of the recognition region.

16. An agent according to claim 15, wherein each of the flanking regions is protected against exonuclease attack.
17. An agent according to claim 16 wherein each of the flanking regions is protected by reverse T.
18. An agent according to any one of the preceding claims, wherein the TNF- α expressing cells are keratinocytes.
19. An agent according to any one of claims 1 to 5, wherein the oligonucleotide is capable of binding to TNF- α mRNA and has a backbone not recognised by RNAase H, which oligonucleotide is attached to a moiety capable of cross-linking the mRNA or cleaving the mRNA, so as to inhibit translation of the mRNA.
20. A composition comprising an agent according to any one of the preceding claims, for use in the treatment of a disorder associated with expression of TNF- α .
21. A composition according to claim 20, which further comprises a duplex cutting enzyme.
22. A composition according to claim 20 or claim 21, which further comprises one or more further agents according to any one of claims 1 to 19, wherein each agent is different from one another.
23. A composition according to any one of claims 20 to 22, wherein the disorder associated with expression of TNF- α comprises an inflammatory skin disorder, cachexia, an autoimmune disorder, meningococcal septicemia, a pulmonary inflammatory disorder, rheumatoid arthritis, septic shock graft versus host disease, and lymphoma.
24. A composition according to claim 23, wherein the disorder associated with expression of TNF- α comprises an inflammatory

- 30 -

skin disorder selected from psoriasis, eczema and ultraviolet erythema.

1/4

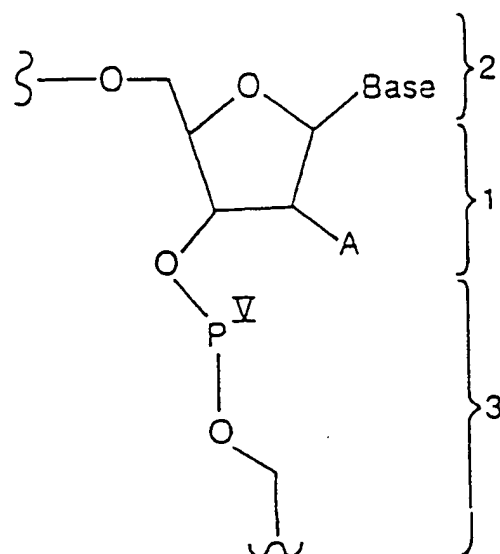


FIG. 1

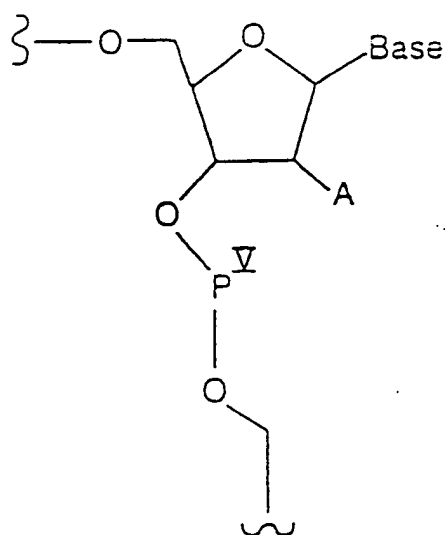


FIG. 2

2/4

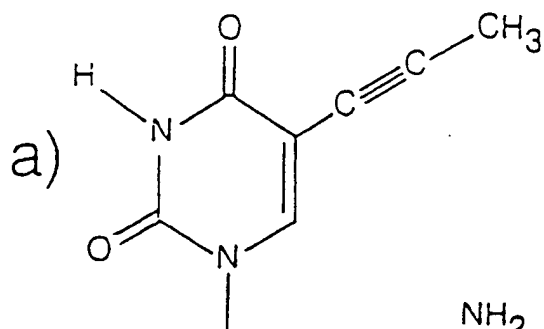


FIG. 3

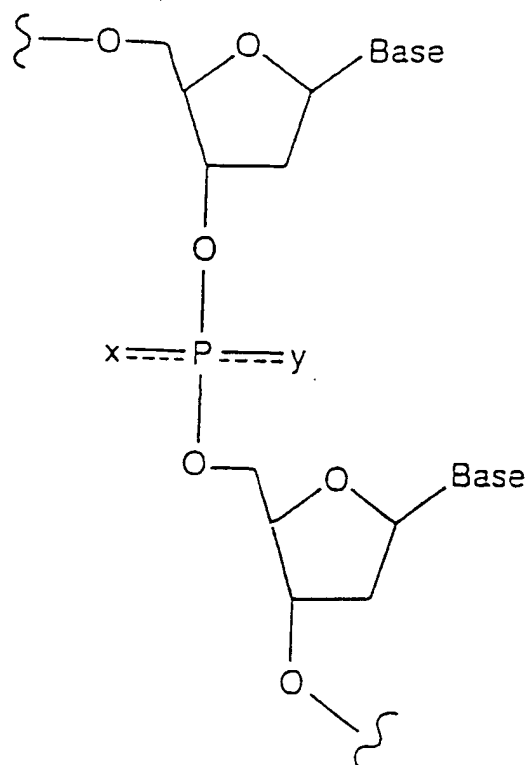
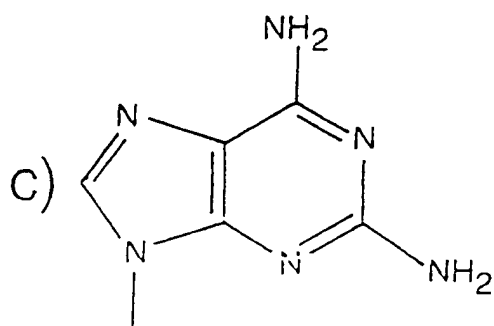
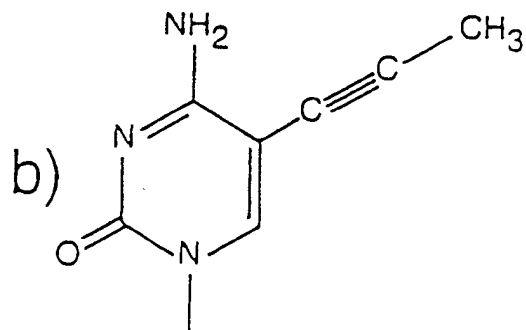


FIG. 4

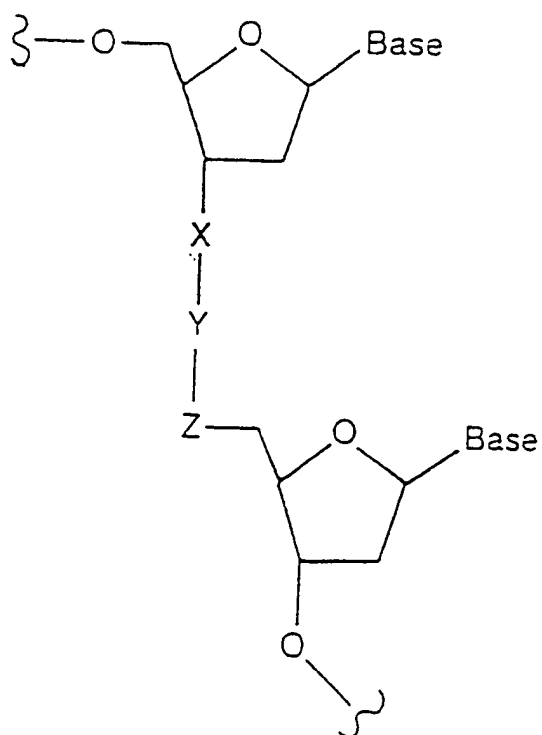


FIG. 5

3/4

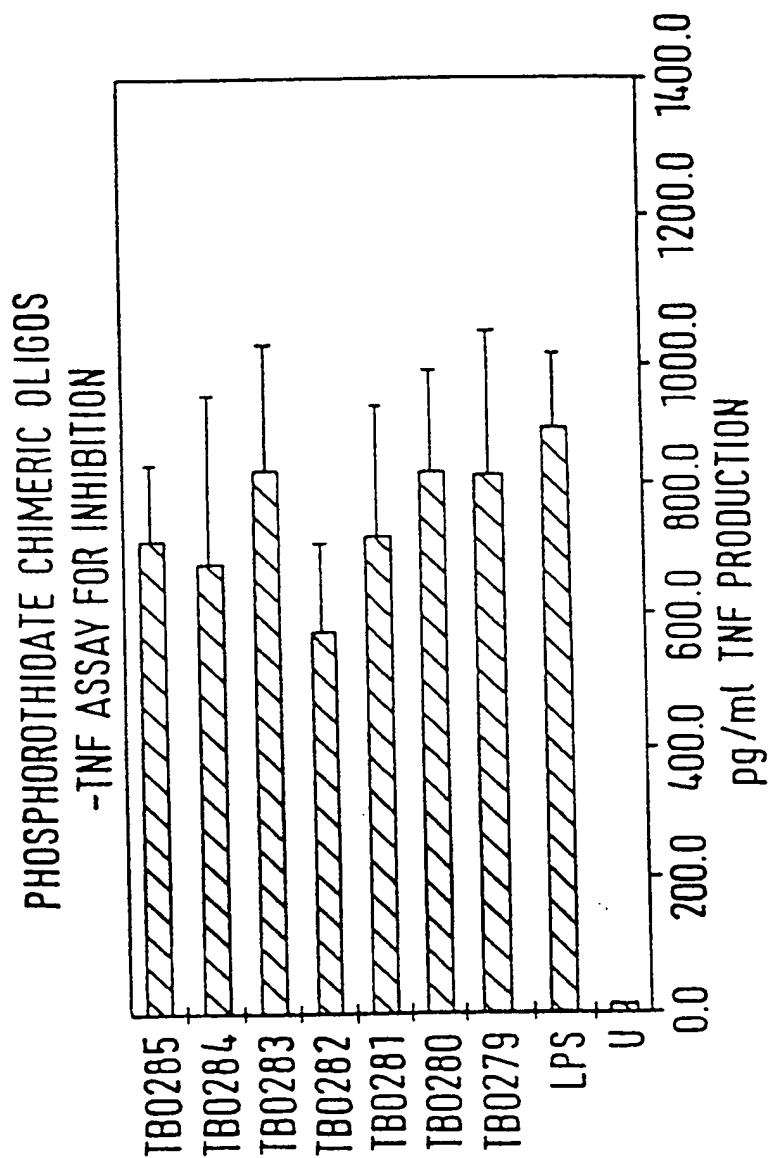


FIG. 6

4/4

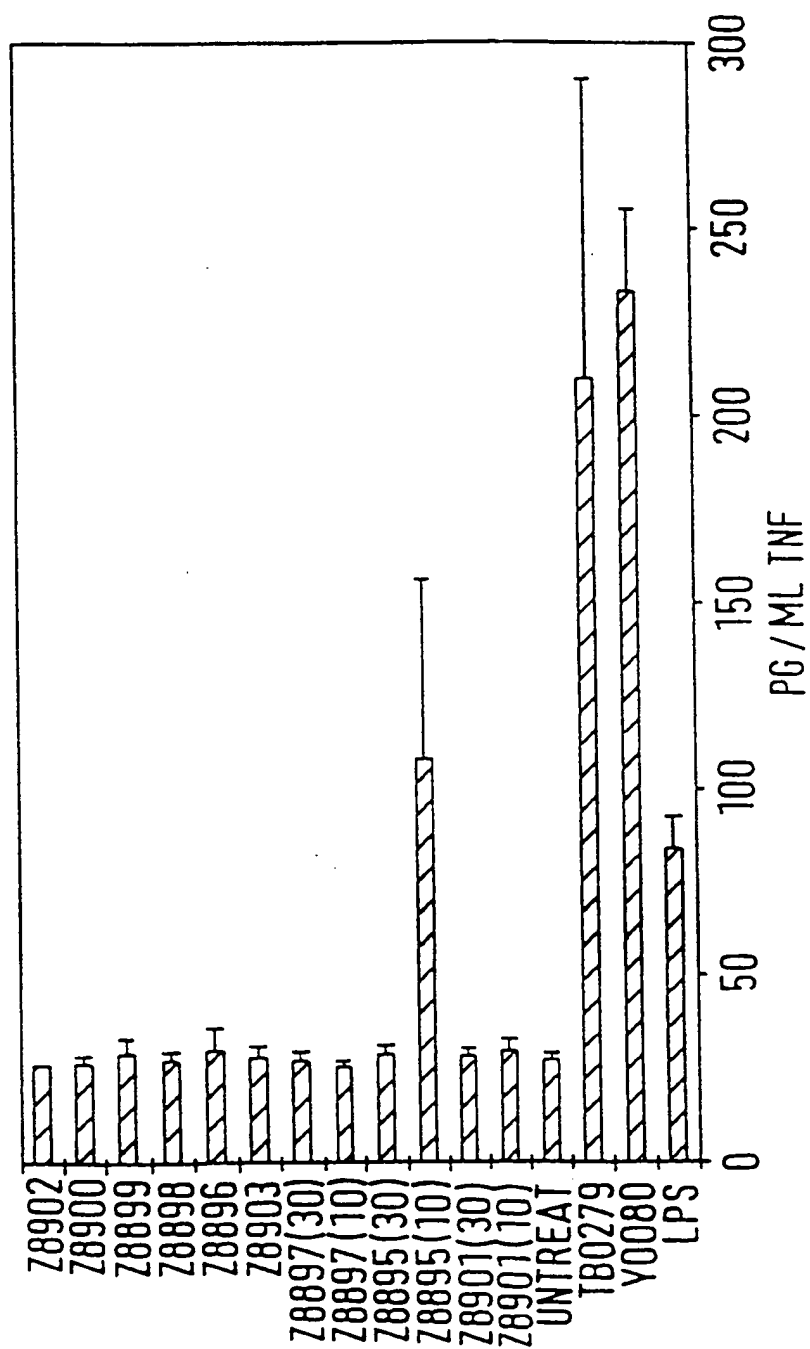


FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/03500

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/11 A61K31/70 C07H21/00 //C12N9/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 10332 A (BRAX GENOMICS LTD ;SCHMIDT GUNTER (GB)) 20 March 1997 cited in the application see page 2, line 8 - page 5, line 33 see examples see page 21, line 8 - page 23 see page 27 - page 29 see claims	1-17,20
Y	---	18,20, 23,24
Y	VASSALLI P: "THE PATHOPHYSIOLOGY OF TUMOR NECROSIS FACTORS" ANNUAL REVIEW OF IMMUNOLOGY, vol. 10, 1992, pages 411-452, XP000646541 see page 415, paragraph 4 see page 421, paragraph 3 see page 424, line 7 - page 425 see page 431, line 10 - page 432, line 11 ---	18,20, 23,24
	--- -/--	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

12 April 1999

Date of mailing of the international search report

22/04/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Andres, S

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/03500

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 00103 A (CHUNG HUN TAEG ;IL YANG PHARM CO LTD (KR)) 5 January 1995 see page 7, line 14 see page 7, line 33 - page 8, line 26 see examples 2,6 see claims ---	1,20
X	EP 0 414 607 A (ROUSSEL UCLAF) 27 February 1991 see page 2, line 29 - line 39 see page 10, line 40 - page 11, line 40 ---	1,20,23
A	HO S P ET AL: "POTENT ANTISENSE OLIGONUCLEOTIDES TO THE HUMAN MULTIDRUG RESISTANCE-1 MRNA ARE RATIONALLY SELECTED BY MAPPING RNA -ACCESSIBLE SITES WITH OLIGONUCLEOTIDE LIBRARIES" NUCLEIC ACIDS RESEARCH, vol. 24, no. 10, 15 May 1996, pages 1901-1907, XP002006548 see the whole document ---	1-21
A	STULL, R. ET AL.: "An in vitro messenger RNA binding assay as a tool for identifying hybridization-competent antisense oligonucleotides" ANTISENSE & NUCLEIC ACID DRUG DEVELOPMENT., vol. 6, 1996, pages 221-228, XP002099425 see the whole document ---	1-3
A	ORTIGAO J F R ET AL: "ANTISENSE EFFECT OF OLIGODEOXYNUCLEOTIDES WITH INVERTED TERMINAL INTERNUCLEOTIDIC LINKAGES: A MINIMAL MODIFICATION PROTECTING AGAINST NUCLEOLYTIC DEGRADATION" ANTISENSE RESEARCH AND DEVELOPMENT, vol. 2, no. 2, 1 January 1992, pages 129-146, XP000573884 see the whole document ---	17
A	WAGNER R W: "GENE INHIBITION USING ANTISENSE OLIGODEOXYNUCLEOTIDES" NATURE, vol. 372, 24 November 1994, pages 333-335, XP002006443 cited in the application see the whole document ---	13
E	WO 99 01139 A (ISRAEL YEDI ;TU GUANG CHOU (US); UNIV JEFFERSON (US)) 14 January 1999 see page 30, line 6 see page 20, line 3 - page 21, line 18 -----	1,4,19, 20,23,24

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 98/03500

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9710332 A	20-03-1997	AU 6939496 A EP 0857208 A	01-04-1997 12-08-1998
WO 9500103 A	05-01-1995	KR 9705347 B AU 6984594 A EP 0737071 A JP 2548507 B JP 7099977 A US 5683988 A ZA 9404185 A	15-04-1997 17-01-1995 16-10-1996 30-10-1996 18-04-1995 04-11-1997 08-02-1995
EP 0414607 A	27-02-1991	FR 2651130 A AT 120201 T AU 642423 B AU 6119090 A CA 2023899 A CN 1049668 A,B DE 69017983 D DE 69017983 T DK 414607 T ES 2069715 T FR 2667202 A GR 3015527 T HU 215242 B IE 68592 B IL 95342 A JP 3091485 A KR 151153 B PT 95067 A,B RU 2066325 C US 5705389 A	01-03-1991 15-04-1995 21-10-1993 28-02-1991 24-02-1991 06-03-1991 27-04-1995 28-09-1995 06-06-1995 16-05-1995 27-03-1992 30-06-1995 30-11-1998 26-06-1996 24-01-1995 17-04-1991 17-08-1998 18-04-1991 10-09-1996 06-01-1998
WO 9901139 A	14-01-1999	AU 8280798 A	25-01-1999